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Nonconventional opioid binding sites mediate growth inhibitory effects of methadone on human lung cancer cells

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ABSTRACT **Methadone was found to significantly inhibit the *in vitro* and *in vivo* growth of human lung cancer cells. The *in vitro* growth inhibition (occurring at 1–100 nM methadone) was associated with changes in cell morphology and viability detectable within 1 hr and was irreversible after a 24-hr exposure to the drug. These effects of methadone could be reversed in the first 6 hr by naltrexone, actinomycin D, and cycloheximide, suggesting involvement of opioid-like receptors and the requirement for *de novo* mRNA and protein synthesis. The inhibitory effects of methadone on the growth of lung cancer cells also could be achieved by the less addictive (+) isomer of methadone. Characterization of the methadone binding to lung cancer cell membranes revealed high-affinity (nM), saturable binding sites for (±)-[^{3}H]methadone, which cross-reacted with ligands for κ, phencyclidine, σ, but not μ, and δ opioid receptors, and the binding characteristics appeared to be different from methadone sites present in rat brain. Methadone decreases cAMP levels in lung cancer cells, but the receptors are not coupled to a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein. We conclude that the lung cancer growth inhibitory effects of methadone are significant, occur at low concentrations, and are mediated by a nonconventional type of opioid binding site distinct from methadone receptors found in the brain.**

In addition to their use in the treatment of pain, opioids have been implicated in the regulation of tumor growth and biology (1–8). Recently, we have shown the presence of biologically active μ, δ, and κ membrane opioid receptors in human lung cancer lines of all histologic types. Opioid agonists selective for μ, δ, and κ ligands were shown to significantly inhibit the growth of these cells in culture (8). In addition, (−)-nicotine reversed the growth inhibition by opioid agonists, suggesting a model in which the normal function of the endogenous opioid pathway would be to suppress tumor growth, while nicotine (from smoking) would overcome this suppressive effect (8).

While examining various opioids for their potential therapeutic value in the treatment of lung cancer, we found the long-acting synthetic narcotic methadone, used for treatment of opioid addiction (9–11), to be a very potent inhibitor of the *in vitro* and *in vivo* growth of human lung cancer cell lines. However, characterization of the methadone binding sites present on lung cancer cells revealed them to be distinct from other opioid receptors and from methadone binding sites present in rat brain membranes.

MATERIALS AND METHODS

Materials. (±)-[*o*,*o*'-$^3H_2$]Methadone (15.98 Ci/mmol; 1 Ci = 37 GBq), nonradiolabeled isomers of methadone, U-50,488H, phencyclidine (PCP), and the (+) isomer of naloxone were donated by the National Institute on Drug Abuse (Rockville, MD). (±)-Methadone hydrochloride (lot 29F0297), naloxone hydrochloride, naltrexone, (−)-nicotine ditartarate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and pertussis toxin were from Sigma. [D-Ala^2,*N*MePhe^4,Gly^5-ol]enkephalin (DAGO) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE) were from Peninsula Laboratories. MK-801 and SKF-10,047 were from Research Biochemicals (Natick, MA).

Cell Lines and Growth Assays. Small cell lung cancer (SCLC) and non-SCLC (adenocarcinoma, squamous, and large cell cancer) cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum as described (12–14). The colorimetric MTT assay was used to measure cell growth (8, 15). Cell growth was also measured by counting viable cells in a hemocytometer by trypan blue dye exclusion after brief trypsinization and by a soft agarose colony formation assay (16). For *in vivo* growth studies, 4-week-old athymic nude mice (N-*Cr-nu*; Harlan–Sprague–Dawley) were injected with 10^6 viable tumor cells (SCLC line NCI-N417) in 0.1 ml of medium into the right flank and tumors were allowed to grow to 3–5 mm diameter. Then, 0.1 ml of sterile saline (control) or methadone (10 mg per kg of body weight, in 0.1 ml of sterile saline solution) was given intraperitoneally daily for 20 days (10 mice per group). Alternatively, mice injected with NCI-H460 (non-SCLC) cells were treated with methadone or saline starting right after implantation (5 mice per group). Tumor diameters were measured daily.

Receptor Binding Assays. Cells were collected during the logarithmic phase of growth, membranes were prepared, and receptor binding assays were carried out as described (5, 8). Membrane protein concentrations were determined (Bio-Rad protein assay kit) and ≈200 μg of membrane protein was used for binding assays. Each experiment was repeated three times. Specific binding was calculated as the difference between total binding and binding in the presence of excess (1 μM) nonradiolabeled methadone. Scatchard plots of the data were evaluated by a modification of the Munson and Rodbard computer program (17).

Intracellular cAMP Measurements. Cells were cultured for 4 days in 24-well plates in 2 ml of medium, and the medium was changed the day before drug treatment. The cells were incubated with the various drugs (100 nM) for 20 min (the time of maximal decrease in cAMP levels) at 37°C, extracts were prepared, and intracellular cAMP levels were measured by a radiometric assay (Amersham kit).

RESULTS AND DISCUSSION

Methadone Inhibits the Growth and Viability of Lung Cancer Cells in *in Vitro* and *in Vivo* Assays. Morphologic effects,

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Abbreviations: SCLC, small-cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAGO, [D-Ala^2,*N*MePhe^4,Gly^5-ol]enkephalin; DPDPE [D-Pen^3,D-Pen^5]-enkephalin; PCP, phencyclidine.

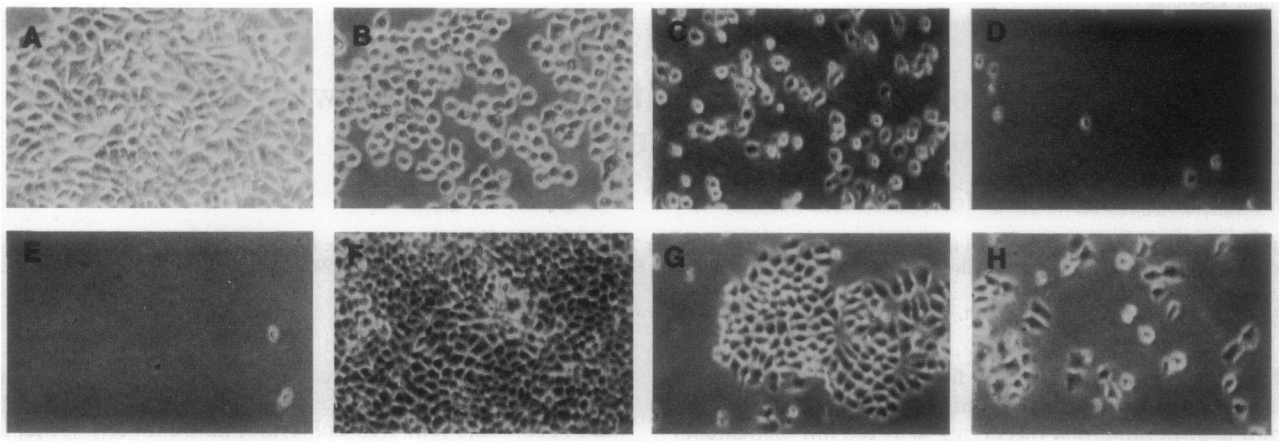

FIG. 1. Effect of methadone on morphology of non-SCLC line NCI-H157 after different times of exposure to 100 nM methadone. Cells before treatment (*A*) or after exposure for 1 hr (*B*), 24 hr (*C*), 48 hr (*D*), or 96 hr (*E*). Cells at 96 hr with no methadone treatment (*F*) or after exposure to methadone for 4 hr (*G*), or 24 hr (*H*). Cells were then washed and grown in methadone-free medium for the remainder of the 96 hr. Note colony growth after 4-hr and no growth after 24-hr exposure. (×54.)

such as cell rounding and detachment from the surface of the culture plates in the case of the non-SCLC cell lines and the loss of refractile properties of cell aggregates in the case of the SCLC cell lines, were seen under phase-contrast microscopy of tumor cells after 1 hr of exposure to the drug and were quite marked after 24 hr of exposure to 100 nM methadone (Fig. 1 *A–F*). Using the 5-day liquid culture colorimetric MTT assay, complete growth inhibition was observed with continued exposure to methadone concentrations of 50–100 nM (Fig. 2). The trypan blue exclusion method also showed that methadone decreased cell viability in 22 of 40 SCLC lines and in 14 of 15 non-SCLC lines, which exhibited ≥75% loss of cell viability at a concentration of 200 nM methadone. Similarly, the soft agarose colony formation assay showed that methadone inhibited the growth of SCLC and non-SCLC tumor cell lines with 50% inhibitory concentrations for 8 lung cancer cell lines of ≈1 μM (data not shown). In addition to the *in vitro* studies, we tested the ability of methadone to inhibit the *in vivo* growth of tumor cells in nude mouse xenografts. Compared to saline, methadone treatment was associated with significant growth inhibition in both treatment schedules (Fig. 3).

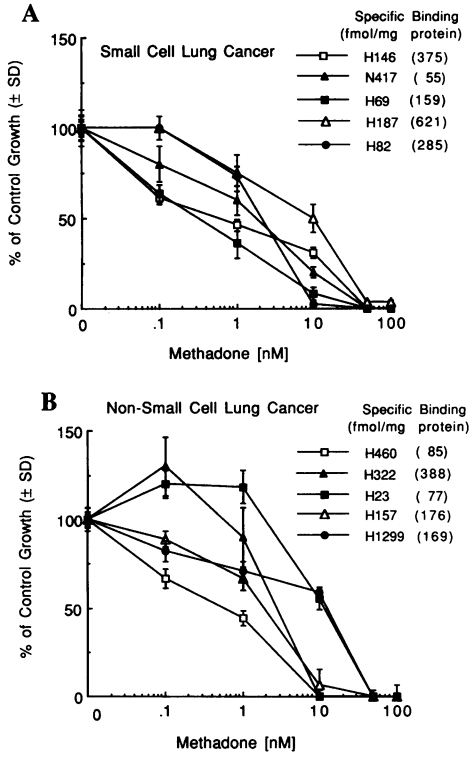

FIG. 2. Effect of methadone on *in vitro* growth of lung cancer cell lines using the liquid culture 5-day MTT growth assay for various SCLC (*A*) and non-SCLC (*B*) lines. Values represent means ± SD of eight culture wells.

Only Brief Exposure to Methadone Is Required for Loss of Cell Viability and This Is Reversed by Cycloheximide and

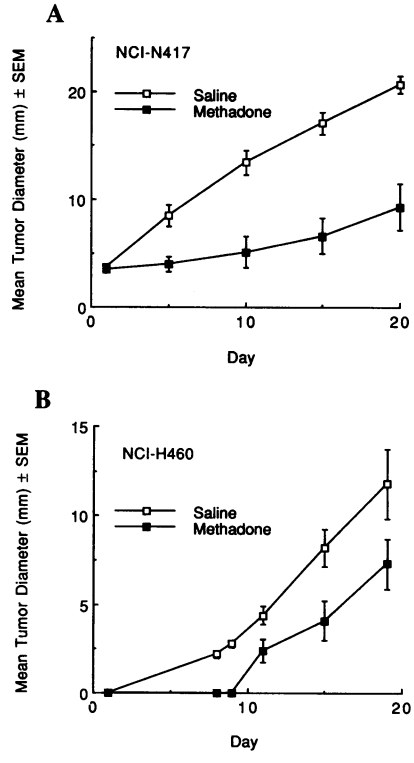

FIG. 3. Effect of methadone on *in vivo* growth of lung cancer cells in nude mice. (*A*) SCLC cell line NCI-N417-implanted mice treated with methadone (10 mg per kg of body weight per day) or saline after tumors developed in mice. Analysis of variance showed $P \leq 0.002$ for days 5–20. (*B*) Non-SCLC cell line NCI-H460-implanted mice treated with methadone or saline at the same time tumor cells were injected into the mice. Analysis of variance showed $P \leq 0.05$ for days 8–14.

Actinomycin D. Cell growth was significantly inhibited with only a few hours of methadone treatment (Fig. 4*A*). After exposure for 4–6 hr to methadone, if the cells were washed and cultured in drug-free medium, subsequent growth of some lung cancer cells was seen, while no regrowth was seen after 24 hr exposure (Fig. 1 *G* and *H*; Fig. 4*A*). These results indicate that the continued presence of methadone is not essential for the growth inhibitory effect after the first few hours of treatment.

Because of the need for only brief exposure to methadone to cause the loss of cell viability, we sought to determine whether *de novo* protein and mRNA synthesis were required for the methadone effect. Treatment of NCI-H157 cells with cycloheximide (5 μg/ml) or actinomycin D (0.05 μg/ml), at concentrations that alone had little effect on trypan blue uptake (<10% trypan blue positive at 6 hr), was able to reverse the loss of cell viability caused by 1 μM methadone (82–99% trypan blue positive with methadone) during the first 6 hr of methadone treatment (Fig. 4*B*). These results suggested that exposure to methadone results in *de novo* synthesis of mRNA and proteins that participate in the growth inhibitory effects of methadone.

Naltrexone Blocks Methadone Growth Inhibition. The long-acting opioid antagonist naltrexone (100 nM) was able to retard the morphologic and cell viability effects of methadone (100 nM) on lung cancer cells during the first 6 hr of treatment, indicating that the methadone growth effects were mediated by opioid-like binding sites (Fig. 4*C*). However, the inhibitory effect of methadone on growth eventually occurred after 24 hr of methadone exposure, even in the continued presence of naltrexone (data not shown). Further support for receptor mediation of the growth effects came from the finding that the inactive (+) isomer of naloxone was unable to reverse the morphologic and growth effects of methadone on lung cancer cells.

Methadone Causes a Decrease in Intracellular cAMP Levels. A variety of properties characterize opioid receptors, including their ability to lower intracellular cAMP levels on activation, inhibition of opioid binding by the guanyl nucleotide GTP, and several protein-modifying agents (18). Exposure of the lung cancer cell lines NCI-H157 and NCI-H187 to 100 nM methadone for 20 min at 37°C caused an ≈50% decrease in intracellular cAMP levels compared to control levels (83 pmol per mg of protein for the NCI-H157 cells and 31 pmol per mg of protein in the case of NCI-H187 cells). The dose–response curve showed that the maximal decrease in cAMP levels occurred at a concentration of 10 nM, with half-maximal inhibition of cAMP levels occurring at 5 nM. Forskolin (10 μM) stimulated intracellular cAMP levels to 200 pmol per mg of protein and methadone decreased this by 64% in the NCI-H157 lung cancer cells. Naloxone (100 nM) when given together with methadone to lung cancer cells actually stimulated cAMP levels (2-fold), again indicating that methadone effects were mediated by an opioid-like receptor.

Pertussis Toxin Reverses Morphine but Not Methadone Effects on cAMP Levels, Tumor Cell Growth, and Viability. Opioids are known to lower intracellular cAMP levels through a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein (18). Thus, we tested whether pretreatment with pertussis toxin had any effect on methadone responses in lung cancer cells. In contrast to our previous results with morphine (8), pretreatment of NCI-H187 cells with pertussis toxin for 3 hr before the addition of 100 nM methadone for 20 min did not reverse the decreases in intracellular cAMP levels observed with methadone alone (methadone treatment resulted in a 48% decrease in cAMP levels, while pertussis toxin pretreatment followed by methadone treatment resulted in a 51% decrease). Pertussis toxin at 100 ng/ml had no effect on the growth of lung cancer cells *in vitro*. While pertussis toxin (100 ng/ml) could effectively reverse the growth inhibitory effect of morphine (in NCI-N417 cells), it was unable to reverse methadone growth inhibition in either NCI-N417 or NCI-H157 cells as measured in the MTT assay (Fig. 4*D*).

Lung Cancer Cell Membranes Exhibit Specific Methadone Binding Sites. Even though methadone has been used clinically for many years, a review of the literature revealed no studies characterizing methadone binding sites in any cell type. Using membrane preparations from a variety of both

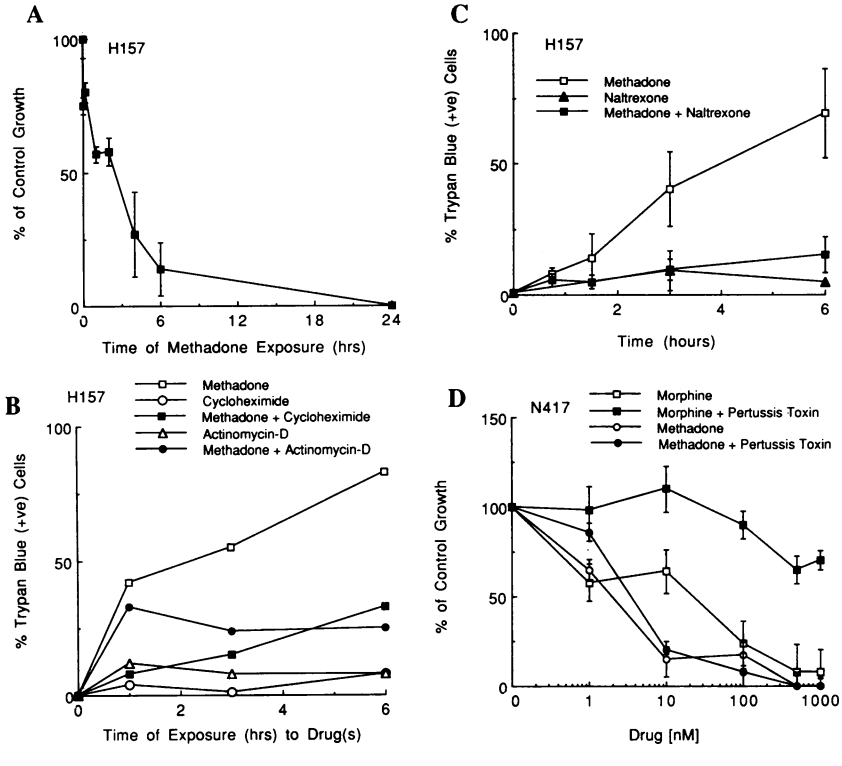

FIG. 4. Characterization of methadone-induced growth inhibition of lung cancer cell lines. (*A*) Effect of time of exposure to 100 nM methadone on the growth of non-SCLC cell line NCI-H157. NCI-H157 cells were exposed to 100 nM methadone for the times indicated, the cells were then washed and refed with methadone-free R10 medium, and the wells were tested 96 hr after the initial plating by the MTT assay. (*B*) Effect of treatment of NCI-H157 cells with methadone (1 μM), cycloheximide (5 μg/ml), actinomycin D (0.05 μg/ml), or combinations of methadone and cycloheximide or methadone and actinomycin D followed by a trypan blue assay after exposure for 1, 3, or 6 hr (% trypan blue positive = dead cells). (*C*) Reversal of loss of viability of NCI-H157 cells (trypan blue assay) caused by methadone (100 nM) with naltrexone (100 nM) during the first 6 hr of methadone exposure. (*D*) The 5-day MTT assay of SCLC line NCI-N417 after treatment with various concentrations of morphine and methadone with and without pertussis toxin (100 ng/ml). For the trypan blue-staining assay, NCI-H157 cells were plated into six-well (3 cm) plates in R10 medium and the drugs were added 18 hr later. Time points [% trypan blue positive (mean of triplicate wells) ± SD] were determined by harvesting the cells from each well and counting in a hemocytometer after trypan blue staining. Control wells with no drug treatment had 1–2 × 10^6 viable cells per well (trypan blue-negative cells) at 6 hr.

SCLC and non-SCLC cell lines (all those shown in Fig. 2) as well as rat brain, (±)-[^{3}H]methadone binding was found to be specific and in the range of 50–900 fmol per mg of protein. (±)-[^{3}H]Methadone binding to intact membranes from SCLC cell line NCI-H187 and non-SCLC cell line NCI-H157 was studied as a function of radioligand concentration. Scatchard analyses revealed specific, high-affinity, saturable binding of methadone to both cell lines and half-maximal binding was achieved at a concentration of ≈5 nM (Fig. 5). Equilibrium binding of (±)-[^{3}H]methadone to SCLC cell line NCI-H187 membranes revealed binding to an apparent single class of high-affinity sites (B_{max} = 878 fmol per mg of protein; K_d = 1 nM; r = 0.97). Scatchard analysis of (±)-[^{3}H]methadone binding to membranes from the non-SCLC cell line NCI-H157 revealed two linear components with K_d values of 0.4 nM and 50 nM for the high- and low-affinity binding components, respectively. Rat brain membranes also showed (±)-[^{3}H]methadone binding to a single class of high-affinity sites (K_d = 3 nM; B_{max} = 3600 fmol per mg of protein).

Methadone Binding Sites on Lung Cancer Cells Are Different from Methadone Binding Sites in Rat Brain Membranes and from Other Opioid Receptors. The methadone binding sites on lung cancer cells and rat brain membranes exhibited different pharmacologic properties. Most of the known opioid drugs and peptides produce their wide spectrum of effects by interacting with at least one of four major receptor types (18). Methadone generally has been considered to behave as a μ agonist, with pharmacologic properties qualitatively similar to those of morphine. Using specific ligands for different opioid receptor types—DAGO for the μ receptor; DPDPE for the δ receptor; U-50,488H for the κ receptor; SKF-10,047 for the σ receptor; and PCP and MK-801 for the PCP/*N*-methyl-D-aspartate receptor—we found that U-50,488H, MK-801, and naloxone were able to significantly displace (±)-[^{3}H]methadone binding to these cell lines, while DAGO and DPDPE were ineffective in displacing (±)-[^{3}H]methadone binding to membrane preparations of NCI-H187 and NCI-H157 lung cancer cell lines (Table 1). In contrast to the lung cancer cells,

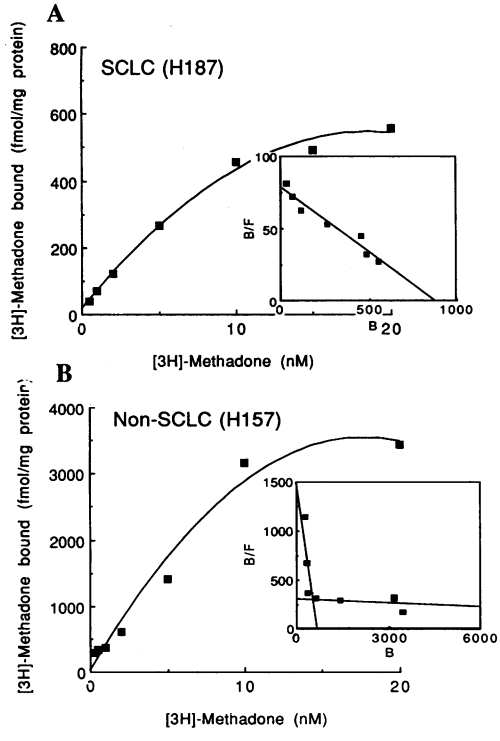

FIG. 5. Saturation binding and Scatchard analysis of (±)-[^{3}H]methadone binding to SCLC cell line NCI-H187 and non-SCLC cell line NCI-H157 cell membranes.

Table 1. Effects of various drugs on (±)-[^{3}H]methadone binding to human lung cancer cell line membranes

Drug	K_d, nM			Specific binding (B_{max}), pmol per mg of protein		
	H187	H157	Rat brain	H187	H157	Rat brain
(±)-Methadone	5	1	1	1.7	0.5	0.6
(−)-Methadone	3	11	4	3.0	0.9	0.5
(+)-Methadone	4	2	1	2.6	0.7	0.3
DAGO (μ)	NDSP	NDSP	5	NDSP	NDSP	0.2
DPDPE (δ)	NDSP	NDSP	8	NDSP	NDSP	0.3
U-50,488H (κ)	7	1	12	0.7	0.5	0.7
MK-801 (PCP)	1	1	10	3.5	0.5	0.7
SKF-10,047 (σ)	4	2	4	2.3	1.4	0.3
Naloxone	8	2	2	2.1	0.5	0.5

NDSP, no displacement seen at concentrations up to 1 μM.

(±)-[^{3}H]methadone binding in rat brain membranes was effectively displaced by the μ, δ, as well as κ and σ, and PCP ligands (Table 1). Further studies showed the lung cancer methadone binding sites to differ from the other opioid receptors. In contrast to the binding of the potent opioid agonist [^{3}H]etorphine to lung cancer cells (8), [^{3}H]methadone binding to membranes from SCLC cell line NCI-H187 and non-SCLC cell line NCI-H157 was not inhibited by various concentrations of GTP, in both the absence and presence of 50 mM NaCl, under the binding conditions used in our assay (Table 2). However, [^{3}H]methadone binding to rat brain membranes was completely inhibited by 100 μM GTP and 50 mM NaCl.

In contrast to methadone binding to rat brain membranes and etorphine binding to lung cancer cells, methadone binding to lung cancer cells appears to be relatively insensitive to protein-modifying agents such as heat, proteinase K, or *N*-ethylmaleimide treatment (Table 2). A similar effect was observed with PCP binding to rat brain membranes, where the binding was only partially inhibited by protein-modifying agents (19). While boiling the SCLC NCI-H187 membrane preparations for 30 min did not inhibit the binding of (±)-methadone, it decreased the ability of MK-801 and U-50,488H to displace (±)-[^{3}H]methadone binding.

Both Stereoisomers of Methadone Are Active. In contrast to other opioid ligands, there is no significant difference in the binding of methadone stereoisomers (18, 20). Similarly, we

Table 2. Effects of GTP and protein-modifying agents on specific binding of (±)-[^{3}H]methadone to membranes from rat brain and human lung cancer cells

Treatment	(±)-[^{3}H]Methadone binding, % of control binding		
	Rat brain	H187	H157
Control	100*	100*	100*
Heat (60°C; 15 min)	69	93	105
Trypsin (10 mg/ml; 30°C; 15 min)	50	65	50
Proteinase K (10 mg/ml; 30°C; 15 min)	31	111	59
NEM (0.5 mM) + DTT (0.25 mM)	58	109	113
GTP			
20 μM	64	83	122
50 μM	56	97	98
100 μM	0	101	99
NaCl (50 mM)	4	105	184
NaCl (50 mM) + GTP (50 μM)	0	85	156

NEM, *N*-ethylmaleimide; DTT, dithiothreitol.
*Rat brain, 513 fmol per mg of protein; H187, 492 fmol per mg of protein; H157, 95 fmol per mg of protein.

found no stereospecificity for receptor binding to lung cancer cell membranes or the rat brain membranes, as both the (+) and (−) isomers of methadone could significantly displace (±)-[^{3}H]methadone binding to these membranes (Table 1). Likewise, the growth effect was not stereospecific since both the (−) and (+) isomers of methadone were found to be equally potent in inhibiting the growth of the SCLC lines (NCI-H187, NCI-N417) and non-SCLC lines (NCI-H157 and NCI-H460) (data not shown). Both isomers of methadone also have been reported to reverse *N*-methyl-D-aspartate toxicity in cortical neurons (21).

Methadone Binding Sites Have Properties Distinct from PCP/*N*-Methyl-D-aspartate Receptors. Since MK-801 and PCP were able to effectively compete for (±)-[^{3}H]methadone binding to these cells, we determined whether L-glutamate, glycine, and D-serine could regulate methadone binding in these cells. These amino acids have been shown to regulate [^{3}H]MK-801 binding to rat brain membranes (22). However, these amino acids did not affect (±)-[^{3}H]methadone binding to lung cancer cells (data not shown). This indicates that binding sites similar to the PCP/*N*-methyl-D-aspartate receptor complex present in rat brain are not involved in the actions of methadone in these cells.

Nicotine Is Unable to Reverse the Effects of Methadone on Lung Cancer Cells. We had previously shown (8) that nicotine was able to reverse the inhibitory effect of other opioids, like morphine, on the growth of lung cancer cells. However, in our current studies, nicotine at doses ranging from 10 nM and 1 μM was unable to significantly reverse the *in vitro* growth-inhibitory action of methadone in a large number of the lung cancer cell lines. Likewise, nicotine had no effect on the binding of methadone to lung cancer cells and nicotine at 100 nM was not able to reverse the decrease in cAMP levels associated with methadone treatment (data not shown).

Methadone Has No Effects on Intracellular Calcium Levels. Some opioids act by directly modulating the voltage-sensitive calcium channels (18). Using the Quin-2 technique (23), we were unable to measure changes in intracellular calcium levels over basal levels in these cells after addition of various doses of methadone (data not shown). In addition, the use of a calcium-channel blocker, Diltiazem, did not block the binding of methadone to its receptor, nor did it reverse the growth inhibitory effects of methadone, suggesting that modulation of calcium levels may not play a primary role in the action of methadone in these cells.

Conclusions. We have found that methadone, a drug already in wide clinical use, has significant growth inhibitory effects on lung cancer cells *in vitro* and *in vivo*, indicating that the antitumor effects of methadone should be investigated in patients. Our results suggest the intriguing possibility that the (+) isomer of methadone, with its lack of significant respiratory depressant activity and 10-fold lower binding to brain membranes than the (−) isomer (18, 20), could be used to treat cancer, while potentially having less-addictive properties. In fact, if methadone is active in patients, its use could be considered at a very early stage of lung carcinogenesis in cigarette smokers. Previously, we found nicotine to reverse the growth inhibitory effects of morphine and DADLE ([D-Ala^2,D-Leu^5]enkephalin) in lung cancer cells (8). In contrast, nicotine was unable to reverse the methadone effect in these cells, indicating the use of methadone instead of morphine in smokers for potentially preventing the development of lung cancer during its very early stages.

In studies using other cancer cell lines, we found that methadone caused the loss of viability in the trypan blue assay of two of two mesotheliomas, two of two colon cancers, two of two breast cancers, one T-cell lymphoma, and five of seven B-lymphoblastoid cell lines, indicating that methadone could be effective in inhibiting the growth of other tumors in addition to lung cancer. However, several human tumor cell lines (especially the adrenal cortical carcinoma line NCI-H295) were resistant to methadone even at concentrations >5 μM. While the mechanism of methadone resistance in these lines is unknown, their existence demonstrates specificity at the cellular level of methadone growth inhibition.

It will be of great interest to characterize the methadone binding sites and to understand the mechanism of methadone-induced growth inhibition in human cancer cells, as this could provide insight into ways to treat cancer. The need for only a brief exposure to methadone and the blocking of the effect with actinomycin D and cycloheximide suggest that methadone acts through a receptor by inducing the synthesis of proteins that participate in the cytotoxic effect. The molecular events that follow activation of the methadone receptor could involve changes in the expression of tumor suppressor or some other class of genes.

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